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Short Research Article

Effect of Sowing Time, Soil Temperature and Inoculum Density on Suppression of Fusarium Wilt in Lentil (*Lens culinaris*)

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Abstract

Several questions remain unanswered on escape mechanism of lentil against Fusarium wilt caused by *Fusarium oxysporum* f. sp. Lentis. Consequently, this study was designed with few parameters including inoculum density, temporal sowing (associated with soil temperature) and its thermal effect with the pathogen. Increased amount of inoculum resulted higher number of diseased plants. However, increased inoculum was proved to be ineffective under cooler condition. Increase in inoculum density numerically enhanced disease incidence at each sowing time (temporal sowing); however, significant difference (p<0.05) increase in disease incidence was detected in the October sown batch. Early sowing associated with high soil temperature (22.6 °C) rendered maximum wilt incidence (97.2%), which is complemented in the laboratory test exhibited by maximum radial growth of the fungus at higher temperature (27 °C). This report suggests development of late sown variety could be a good option to suppress this disease.

1. Introduction

Unlike leaf infection, different mechanisms are operated for infection in roots under different pathosystems (Sesma and Osbourn, 2004; Raaijmakers et al., 2008; Marchetti et al., 2010). This element of infection draws attention, because the altered plant organ injury may correspond to dissimilar damage mechanisms which ultimately lead to crop loss. The type of crop loss is directly associated with quantitative reduction of harvest in case of wilt disease of cool season legume like lentil (*Lens culinaris* Medikus), caused by soil-borne fungus *Fusarium oxysporum* f. sp. *lentis* (*FOL*). This typical feature of this disease has made present study more vivid because percent infected plant is directly proportional to crop yield loss.

Lentil wilt epidemics can occur during the early stage of crop cycle; however, the wilt injury can take place at any developmental stage of the plant under various climatic conditions (Turk et al., 2004; Abd-Allah and Hashem, 2006; Akem and Bayaa, 2006). Effect of several agronomic practices like sowing date, sowing depth and soil types are in general associated with manipulation of micro-environment which influences lentil wilt disease. These changes are complex and often interrelated, because they affect both host and root pathogens. Some factors may affect the lentil plant negatively and the fungus positively, leading to an apparent increase in lentil wilt. In other way, these may affect both the lentil and fungus positively, leaving the resultant disease increase or decrease in a matter of speculation.

Fusarium wilt is a monocyclic disease. The initial inoculum level is very important for determining the incidence and severity of the disease. The pathogen survives as chlamydospore and dormant mycelium in infected plant debris and in soil. The fungus can also be seed-borne either as systemic infection inside the seeds or as a contaminant on the seeds. Sowing time affects wilt incidence because it determines the growth stage of the crop and an optimum or near-optimum temperature for fungal growth. Warm soil temperature (20-30 °C) and dry soil conditions (having 25% water holding capacity) are the two most important environmental factors favouring development of the disease (Khare, 1981). Hwang et al. (2000) also reported that sowing dates were found to play a vital role in the development of lentil wilt disease. In a controlled environment study, the impact of temperature on infection of lentil seedlings by *Fusarium* species, associated with rot and brown discolouration symptoms in root, the disease was severe at warm temperature (20-27.5 °C) and declined in cooler soils. Therefore, the current study was formulated and aimed to

examine the difference in wilt incidence under distinct planting time, which is associated with varied soil temperature. Effect of inoculum level under a given sowing time on lentil wilt incidence was also observed.

2. Materials and Methods

2.1. Isolation and identification of causal organism

Roots of wilting lentil plants were used for fungus isolation. They were washed carefully under tap water to remove the adhering soil particles. The washed roots were cut into small pieces of up to 2 cm length and surface sterilized by 1% sodium hypochlorite for 30 seconds and then rinsed with distilled water for four times. Surface sterilized roots were placed onto 1.7% malt extract agar (MEA) medium in Petri plates and incubated at 25 °C. After 6-7 days, the fungal isolates appearing on the root pieces were identified and transferred to 1.7% MEA-plates for purification. Pure culture of fungus was obtained using single spore or hyphal tip technique. The fungal isolates were then identified according to Booth (1985); pure culture was kept in refrigerator at 4°C for further studies.

2.2. Inoculum preparation

According to modified method of Saju et al. (2002), one-litre flasks were filled with 100 g of sorghum seeds (*Sorghum bicolor*) and 200 ml of water and autoclaved for 1 hr. Flasks were seeded with four agar plugs (5-mm dia.) removed from the outer margins of an FOL colony. Jars containing the inoculated mixture were shaken periodically to ensure complete colonization of the grain. After incubation, the infested grains were air dried and stored at 4 °C until needed. Prior to storage, the infested grains were ground in a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) using a 25-mesh screen to obtain uniform size of milled-grains (inoculum of the pathogen). The viability of inoculum was assayed on PCNB agar to determine the number of colonies formed per gram of inoculum (colony-forming units: cfu). The inoculum provided an initial population density of 3×10^6 cfu g^{-1}. This information was used to calculate the weight of milled inoculum required per kilogram of soil mixture for the controlled-environment studies.

2.3. Pathogenicity test

Plastic pots (25 cm diameter) were filled with sterilized soil (autoclaved at 15 psi for 1 hr in polypropylene bags) and infested with fungal inoculum at the rate of 3% of soil weight. Pots were irrigated and left for 7 days to ensure the establishment of the FOL isolate in the soil. Seeds of lentil variety PL-5, susceptible to Fusarium wilt, were disinfested by dipping it in 1% sodium hypochlorite for 2 min and then washed several times in sterilized distilled water. Thereafter the seeds were sown in the infested pots at the rate of 4 seeds pot^{-1}. Pots containing sterile soil mixed with grain free of any fungi were sown similarly with lentil seeds at the same rate to be used as a control treatment. All pots were irrigated after soil infestation and later when it is needed.

2.4. Soil inoculation

Plastic pots (17.5 cm dia.) were filled with the inoculum-soil mixture (Paulkar and Raut, 2004); and incubated for 3 days before sowing inside a glass house. Inner surface of the pots was disinfected with 1% sodium hypochlorite. The field soil was autoclaved at 15 psi (121 °C) for 20 min. for two successive days. Milled sorghum colonized inoculum were mixed with sterilized soil (weight basis) with three different proportions i.e. 1:9, 2:9 and 3:9. A non-amended control treatment was included, for this, twice-autoclaved; non-colonized sorghum was utilized.

2.5. Plant material and soil temperature

Lentil variety PL-5, susceptible to Fusarium wilt, was obtained from the Breeder Seed Processing Centre (BSPC), Pantnagar. Sowing dates were 1st October, 1st November and 1st December in 2011. Before sowing, seeds were placed in a 1% sodium hypochlorite solution (weight basis) for 40 seconds and rinsed thrice with sterilised distilled water. Nine seeds were sown in each pot and placed in a net house. Sprinkles of water sprayed over seeded pots immediately after sowing. The seeded pots were irrigated in the late evening using a hose additionally at 2-day interval. Six seedlings pot^{-1} were maintained by uprooting extra seedlings at 10 days after sowing. Soil temperature (5 cm depth) was recorded thrice a day (11 am, 2 pm and 5 pm) using a soil thermometer. Mean soil temperature was 30.2, 26.5 and 16.1 °C for October, November and December, respectively.

2.6. Experimental design

Two pots were included (6 plants pot^{-1}) in an elementary unit. Each elementary unit considered a replication of a given planting date×inoculum density combination. The two-factorial experiment was performed according to a split-plot design with three replications, whereby planting date was considered as the main-units and inoculum density was implemented as the sub-units.

2.7. Data acquisition

Wilt incidence scored two times (28 and 49 days after sowing) for each batch. Computation of wilt incidence was performed as the number of diseased plants divided by the total number of established plants within each pot. Plant was counted diseased once the plant was stunted or dead and exhibited the typical wilting and yellowing associated to Fusarium wilt of lentil. At final assessment, the root cortex was examined for the presence of fungal hypha resulting discolouration and necrosis (Figure 1). Necrotic root cortex tissue sampled from each lentil planting was placed on 1.7% MEA to validate the existence of FOL.

2.8. Analysis of data

Effects of planting date, inoculum density, and their interactions

were assessed using analyses of variance (ANOVA) with a split-plot design. Analyses were performed using the software CropStat 7.2 (IRRI, Philippines).

3. Results and Discussion

A previous attempt for management of a cool season legume using bio control fungus demands further work for disease escape ability of plant against soil-borne pathogen (Ghatak et al., 2010). Therefore, this investigation was designed to evaluate different components which can be adopted by the lentil growers against Fusarium wilt. The effects of sowing time, inoculum density, and their interactions on the incidence of lentil wilt were investigated. Effect of incubation temperature on radial growth of FOL on MEA was also studied following different temperature regimes.

3.1. Sowing time and Fusarium wilt

Lentil wilt disease development was significantly affected by sowing time (p<0.01, 0.01; Table 1). In this investigation, wilt incidence was recorded twice for each tested inoculum density at 4- and 7-wps (weeks post sowing) within each of the three sowing time for three batches (Figure 2). Our results advocate for delayed sowing that reduced lentil wilt incidence for both assessments. However, at final observation of batch 1 (sowing in October) only resulted in significantly high disease incidence under any of the categories of inoculum density. November and December sowings (batches 2 and 3) were not significantly differed development.

Within each sowing time, significant difference in wilt incidence was noticed with stronger effect at 7-wps (Table 1). Sowing time is considered as one of the limiting factors for disease incidence (Akem et al., 2004; Landa et al., 2004). Sowing in December (batch 3) was more appropriate to minimise the disease other than October and November (Figure 3). Hwang et al. (2000) found that sowing dates of lentil plants play vital role in the development of lentil root disease. Results of the present study confirmed that the choice of sowing time influences the development of Fusarium wilt, as reported by other workers that winter sowing of chickpea contributes to control of Fusarium wilt which results in increased yield. However, the efficacy of winter sowing as a management practice for Fusarium wilt is influenced by several factors in the pathosystem, and may be significantly reduced under changing climatic scenario (Navas-Cortes et al., 2000b). Sowing in October (batch 1) produced maximum diseased plants followed by sowing in November (batch 2) and December (batch 3), respectively (Table 2).

3.3. Impact of temperature on Fusarium oxysporum f. sp. *lentis*

The effect of temperature on incidence of Fusarium wilt of lentil was monitored. Our findings advocate for the higher soil temperature the higher wilt incidence in lentil. The maximum mean soil temperature was observed in October (Table 3) where highest wilt incidence was recorded (Table 2; Figure 2). This observation was further confirmed under laboratory condition whereby activity of the fungal mycelium was found to be more at high temperature (Figures 1 and 4). Effect of incubation

Table 1: Analysis of variance table evaluating the effects of sowing time, inoculum density, and their interactions on the incidence of Fusarium wilt of lentil artificially infested by *Fusarium oxysporum* f. sp. *lentis*

Sources of variation[x]	Fusarium wilt incidence						
	4 weeks post sowing				7 weeks post sowing		
	df	F	P>F[z]		F	P>F	
Replication (R)	2	2.77	0.176	NS	0.68	0.558	NS
Sowing time (ST)	2	45.76	0.003	**	30.31	0.005	**
R×ST	4	0.74	0.584	NS	1.34	0.312	NS
Inoculum density (ID)	2	17.58	0.012	*	10.64	0.027	*
ST×ID	4	0.36	0.831	NS	1.18	0.368	NS

[x]: Sources of variation. Sowing time: October, November and December. Inoculum density: ratio of infested seed and soil. Analysis was performed with arcsine transformed value; [y]: Degrees of freedom; [z]*, ** and NS=significant at p< 0.05, p<0.01, and non-significant, respectively.

Table 2: Fusarium wilt incidence of lentil sown in October, November and December among different levels of inoculum density

Infested sorghum seed:soil	October		November		December	
	Disease incidence (%)		Disease incidence (%)		Disease incidence (%)	
	Range	Mean	Range	Mean	Range	Mean
4-wps						
1:9	50.0-58.3	55.6[a]	33.3-58.3	47.2[a]	16.7-41.7	27.8[a]
2:9	58.3-75.0	63.9[ab]	50.0-58.3	52.8[a]	25.0-41.7	33.3[a]
3:9	66.7-83.3	75.0[b]	58.3-66.7	61.1[a]	33.3-41.7	36.1[a]
7-wps						
1:9	66.7-91.7	77.8[a]	33.3-58.3	50.0[a]	33.3-50.0	41.7[a]
2:9	75.0-91.7	86.1[a]	58.3-66.7	61.1[a]	58.3-66.7	58.3[a]
3:9	91.7-100.0	97.2[b]	66.7-66.7	66.7[a]	58.3-66.7	63.9[a]

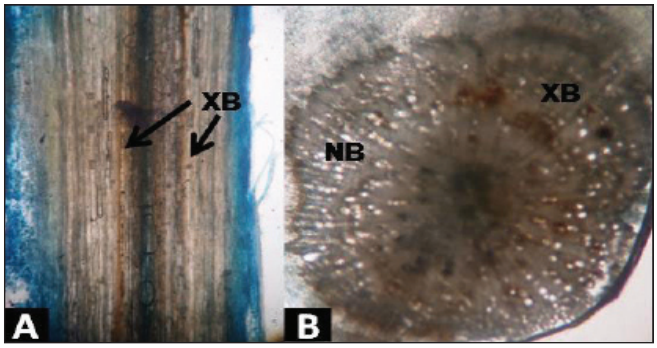

Figure 1: Fusarium infected lentil root: a- longitudinal section and b- transverse section showing xylem blocking (XB) and non-blocking (NB) region

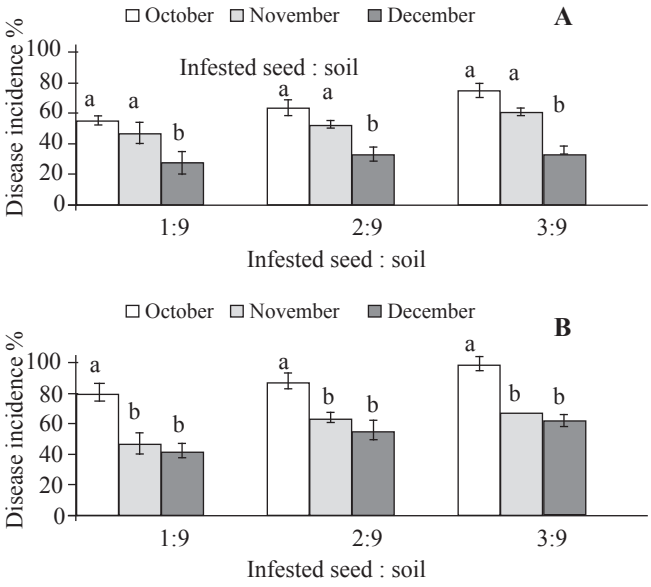

Figure 2: Effect of temporal sowing according to a level of inoculum density on Fusarium wilt incidence of lentil on A, 4-week post sowing; B, 7-week post sowing. Error bars are standard error of the mean. Common letter of bars produced in a particular inoculum density is not significantly different according to the LSD test. LSD for combination of planting time and inoculum load at $p>0.05$ is 0.157 and 0.196 for A and B, respectively.

temperature on radial growth of *FOL* on MEA medium was studied. Mycelial growth of the tested fungus was recorded on MEA medium. Effect of different temperature regimes against *FOL* was tested.

Maximum radial growth was observed on 27 °C both on 4-days after inoculation (dai) and 7-dai (Figure 4). The radial growth was found to increase with increasing temperature up to 27 °C. Polynomial regression analysis displayed reduced radial growth both at 4-dai (r^2=0.69) and at 7-dai (r^2=0.67) with increasing temperature 27 °C. Lowest temperature (15 °C) produced smallest radial growth of the tested fungus. The present findings are in accordance with earlier observations of Landa et al. (2001) who reported that optimum growth of *F. oxysporum* f. sp. *ciceris* was at 24.5-28.5 °C. Mina and Dubey (2010) also reported that maximum colony diameter of *Fusarium oxysporum* was observed at 28 °C, followed by 30 and 35 °C. This temperature range also favoured for maximum sporulation of the pathogen in culture. Hubbard and Gerik (1993) reported that an isolate of Fusarium wilt of lettuce grew with maximum growth at 28 °C.

Soil temperature was recorded at 5 cm depth from sowing to first disease rating, from first to final rating, and from sowing to final rating (Table 3). The mean soil temperatures from sowing to first rating were 24.4 °C, 18.9 °C and 14.6 °C; first rating to final rating 20.1°C, 15.8°C and 13.8°C, and from sowing to final rating 22.6 °C, 17.6 °C and 14.3 °C for batch 1, batch 2 and batch 3, respectively. In each case, highest temperature was observed in the month of October. Soil temperature recorded for

Table 3: Mean soil temperature during different stages of the experiment

Observation	Time[y] (days)	October	November	December
Sowing to first rating	28	24.4	18.9	14.6
First rating to final rating	21	20.1	15.8	13.8
Sowing to final rating	49	22.6	17.6	14.3

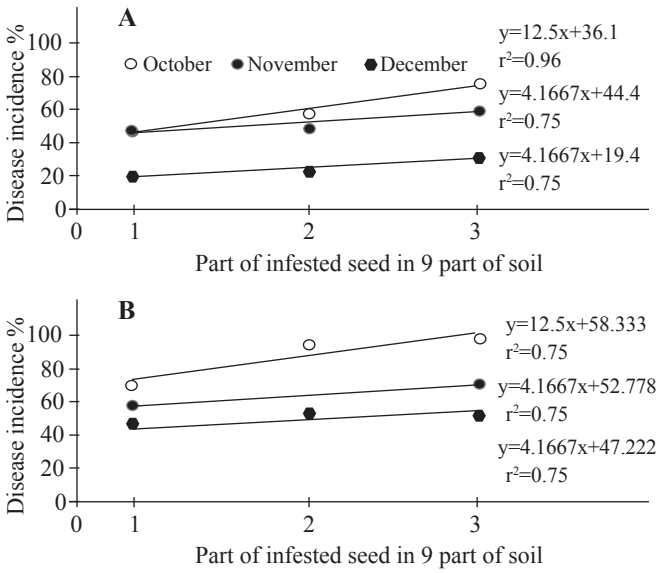

Figure 3: Relationship between inoculum density and temporal planting on Fusarium wilt incidence of lentil on A, 4-week post sowing; B, 7-week post sowing.

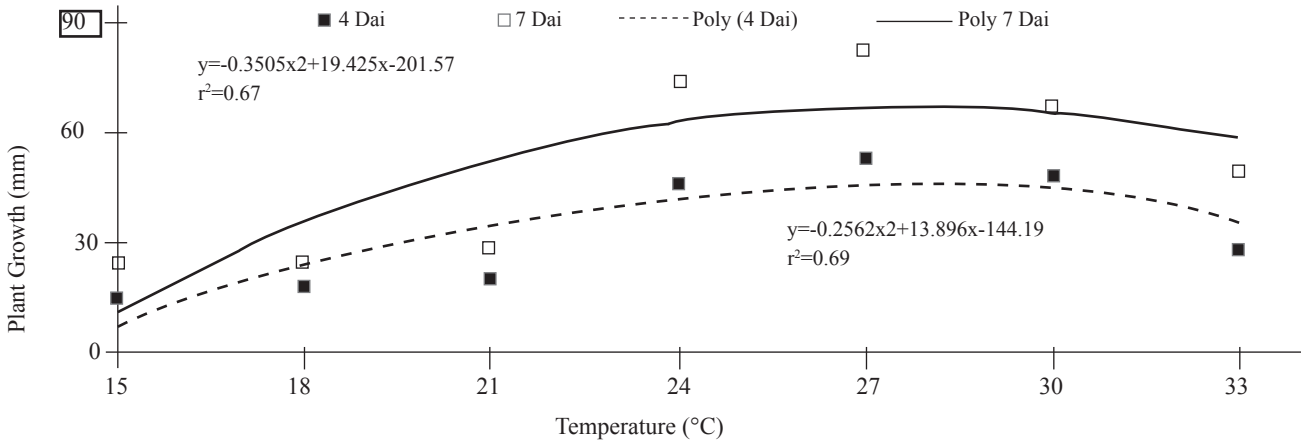

Figure 4: Relationship between radial growth of *Fusarium oxysporum* f. sp. *lentis* on malt extract agar and incubation temperature

October sowing was found to be apparently more favourable for disease development, as lowest soil temperature was recorded in December sowing rendering minimum wilt incidence. In a related study of chickpea, symptoms of Fusarium wilt were less severe from 10 to 20 °C than from 25 to 30 °C (Bhatti and Kraft, 1992). Similarly, a constant temperature of 35 °C was most favourable for disease development on chrysanthemum (Gardiner et al., 1987). In present experiment, delay in the date of sowing from 1st October to 1st December minimizes the development of Fusarium wilt may be because of decrease in soil temperature.

In our study, a greater incidence was recorded when mean soil temperatures were between 22 and 24 °C compared with disease levels at 14.3 and 17.6 °C. In the same way, Fusarium wilt of carnation, caused by *F. oxysporum* f. sp. *dianthi*, was most severe at 25 to 26 °C, whereas plants below 18 °C remained symptomless (Ben-Yephet and Shtienberg, 1994). Working on Fusarium wilt of banana caused by *F. oxysporum* f. sp. *cubense*, Peng et al. (1999) reported an increase in disease severity as temperature increased from 24 to 34 °C, whereas at 14 °C, no wilt symptoms were evident even though the pathogen could be recovered from within banana plant tissue. Through modelling, Navas-Cortes et al. (2007) reported positive correlation between wilt severity and soil temperature can affect the relative resistance response of certain pulses against *F. oxysporum* under field conditions. This demonstrates the importance of temperature and date of sowing for the management of Fusarium wilt of lentil.

3.2. Inoculum density and Fusarium wilt

Increasing the amount of initial inoculum enhances disease severity of Fusarium wilt and early onset of disease is occurred (Bhatti and Kraft, 1992). A significant effect of inoculum density in wilt development for both of the assessments was detected through analysis of variance (Table 1). The result indicates greater possibility to wilt appearance in a field where population of *FOL* is high. However, the population could be minimised with delayed sowing, which is supported by the non-significant interaction of sowing time and inoculum density ($p>0.05$). Increased inoculum density is ineffective under cooler condition (Table 2). Increased inoculum density provided significant effect only in batch 1 (sown in October) when relatively high soil temperature was recorded (Table 3). This information was further confirmed by establishing relationship between inoculum density and temporal planting on Fusarium wilt incidence of lentil (Figure 3). Higher wilt incidence was generated with increase in inoculum density but a pattern of lag phase of a typical sigmoid curve was detected in the batch 1 when mean soil temperature was highest among the three-month observation.

In October sowing, significantly higher wilt incidence was recorded at 4-wps for the medium (2:9) and highest (3:9) inoculum density compared to the lowest inoculum density (1:9). However, no significant difference was observed for the lowest and medium inoculum density at 7-wps among all sowing months (Figures 2B and 3B). Remarkably, highest inoculum density (3:9) in October sowing produced significantly higher wilt incidence for both 4- and 7-wps (Figures 2 and 3). Fusarium wilt incidence was significantly affected by inoculum density ($p<0.01$, 0.05 Table 1); similar finding was reported by other workers working on Fusarium with different pathosystems (Landa et al., 2001). Navas-Cortes et al. (2000a) observed an exponential reduction of the time for disease onset with increasing the inoculum density of pathogen causing Fusarium wilt in chickpea. Degree of wilt incidence can be specific to the race of the pathogen (Navas-Cortes et al., 2000a). Some races of *F. oxysporum* f. sp. *ciceris* may require higher amount of primary inoculum, however, numerous races have been reported that can manifest high wilt incidence with small amount of primary inoculum.

4. Conclusion

Choice of sowing date influences development of the Fusarium wilt in lentil. Our experiment advocates for delay sowing to

escape a period favourable to *FOL* growth, therefore, escape the phase when *FOL* is more aggressive. It was demonstrated that incubation temperature and inoculum density of *FOL* firmly interrelated in altering the suppression of wilt in lentil. Winter sowing as a management practice for Fusarium wilt is influenced by several factors in the pathosystem, and may be significantly reduced under changing climatic scenario.

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